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Maintenance of Ectomycorrhizal Fungi

HELVI HEINONEN-TANSKI and TOINI HOLOPAINEN
Department of Environmental Sciences, University of Kuopio, PO Box 6, SF 70211 Kuopio, Finland

I. Introduction

Detailed accounts of the isolation, cultivation and maintenance of ectomycorrhizal fungi have been given in the article by Molina and Palmer (1982) and many of their procedures have been adopted in our work. In addition, some other methods used in studies of ectomycorrhizal fungi of coniferous trees of Scandinavia, e.g. Scots pine (*Pinus sylvestris* L.) and to a lesser degree on Norway spruce (*Picea abies* L.) are described.

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II. Cultivation of ectomycorrhizal fungi

A. Establishment of a collection of mycorrhizal fungi

When starting a collection of ectomycorrhizal fungi it is desirable to obtain cultures from well-known collections or from other ectomycorrhiza research workers. Cultures are in most cases obtained as agar slants which can be easily sent by post or carried on the person. In cold climates special care must be taken to ensure that mycorrhizal cultures are not frozen during transport.

The isolation and cultivation of ectomycorrhizal fungi is greatly facilitated by the use of a laminar flow-chamber. Apparatus and instruments useful for isolation purposes are Petri dishes containing suitable nutrient agar (see below), sterile scalpels, thick double or triple cultivation loops (Schaffer, 1982) and metal forceps which can be sterilized by flaming in ethanol before each isolation or subculture.

Suitable isolation media include modified Hagem's agar (Modess, 1941) and modified Melin–Norkrans' agar (Marx, 1969). The main difference between these media is that Melin–Norkrans' agar contains more thiamine while Hagem's agar contains more ammonium salt and is nutritionally more versatile than the synthetic modified Melin–Norkrans' agar. Half of the carbon source in the former medium comes from malt extract, the content of which varies but usually contains more than 50% maltose and approximately 40% other carbohydrates, some protein and ash (Oxoid, 1982). Both of these media are recommended when isolating new strains of ectomycorrhizal fungi. The content of modified Hagem's agar is: KH_2PO_4 0.5 g, NH_4Cl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3$ (1%) 0.5 ml, glucose 5.0 g, malt extract 5.0 g, thiamine HCl 50 μg, aureomycin 35 mg, agar 15 g and water 1000 ml. The content of the modified Melin–Norkrans' medium is KH_2PO_4 0.5 g, NH_4Cl 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $FeCl_3$ (1%) 0.5 ml, $CaCl_2$ 0.05 g, NaCl 0.025 g, glucose 10.0 g, thiamine HCl 100 μg, aureomycin 35 mg, agar 15 g and water 1000 ml. The media are autoclaved for 15 min at 121 °C and the pH should be 4.5–5.0 for Hagem's agar and 5.7–6.2 for Melin–Norkrans' agar. If the agar medium is too soft, the amount of agar can be increased to 20–25 g litre^{-1}. Because cultivation times are often quite long, it is important that the agar does not dry out and 20–25 ml of media per 9 cm Petri dish is often suitable.

The antibiotic aureomycin can be added to the media at a concentration of 35 mg litre^{-1} but is omitted when pure cultures are being maintained. Benomyl (1 mg litre^{-1}) is sometimes used against air-borne fungal contaminants (Marx, 1969), with or without aureomycin.

1. Isolation from sporophores

Fresh isolates can be obtained from mycorrhizal roots or from sclerotia in boreal coniferous forests during the growing season. Many fungi are easily isolated in late summer or autumn when ectomycorrhizal fungi appear on the soil as sporophores.

The isolation of mycorrhizal fungi is easiest from a sporophore, which of course must be identified correctly. Palmer (1971) pointed out that it is important to make careful descriptions of the growth site, host plants and if possible to take colour photographs of the site and the sporophores present in addition to the preservation of reference specimens, which can be dried (Molina and Palmer, 1982). Kendrick and Berch (1985) advise depositing the reference specimen in an internationally recognized herbarium listed by Holmgren *et al.* (1981).

The sporophores collected should be young, healthy and undamaged. Material containing mites and insects should be rejected. The sporophores should be wrapped in paper and rapidly transported to the laboratory, ideally during the same day without additional damage. Isolations should be made soon after arrival in the laboratory. If the rapid transport of sporophores is not possible, the use of a portable field cultivation chamber (Molina and Palmer, 1982) is recommended. The chamber used by Molina and Palmer consisted of a 15 cm × 40 cm chamber of open frame, with a plexiglas lid and sides and bottom of plywood.

The sporophores should be pre-cleaned before being moved to the inoculation laboratory. In most cases tissue taken from the inner parts at the junction between the stipe and the cap of the sporophore has been found to be most successful. The inoculum can be removed after breaking the sporophore carefully by hand to expose inner surfaces which are cut as small pieces using a sterile scalpel. The inner surfaces must not be touched during the preparation and contamination from the outer surfaces must be avoided. Three or four cubic pieces with dimensions of approximately 1–3 mm are cut with a sterile scalpel and placed on agar in each Petri dish. Normally several inoculum pieces can be readily obtained from the same sporophore.

2. Isolation from basidiospores

The mature hymenal surface can sometimes be taken for isolation of ectomycorrhizal fungi using the basidiospore-drop technique of Palmer (1971). A piece of hymenium is attached using agar or petroleum jelly

to the surface of a glass tube so that spores can fall readily onto the agar slant placed just below, but not in contact with, the hymenium. The lid of a Petri dish can also be used, the hymenium again being arranged so that spores fall onto agar in the lower half of the Petri dish.

3. *Isolation from mycorrhizal rootlets*

Ectomycorrhizal fungi can be isolated from mycorrhizal rootlets. This is the only available technique in the case of those fungi that do not produce sporophores. Fresh roots are collected and washed to remove adherent soil. Lateral, young, fine root organs containing mycorrhiza are surface sterilized for 15–30 s with hydrogen peroxide (30%, v/v) or for 3–6 min with sodium hypochlorite (with 6–8% active Cl) and washed repeatedly in sterile water to remove all traces of the sterilant. The material should not be moved to the cultivation laboratory until it has been through the sterilization procedure. The organs, cut into smaller pieces, are then placed onto an appropriate agar medium (see above) for incubation. Mercuric chloride used as sterilant and recommended in the older literature should be avoided because of its toxicity. Contaminants from soil are common in spite of careful precautions. In many cases also the mycorrhizal fungus does not grow readily on artificial medium or where growth does occur, it is too slow for experimental purposes.

4. *Isolation from sclerotia*

Some fungi, for example *Cenococcum geophilum*, can also be isolated from sclerotia. Sclerotia are sieved from soil, washed, surface sterilized and carefully rinsed (Molina and Palmer, 1982). Similar procedures can be applied to structures such as rhizomorphs produced by some ectomycorrhizal fungi (Kendrick and Berch, 1985).

5. *Incubation of primary isolations*

In most cases hyphae develop during an incubation time of 1–7 weeks at 17–25 °C. Drying and contamination can be minimized by sealing the rims of the closed Petri dishes with Parafilm. Growth can be followed by eye or by stereo-microscope after 2–3 days and then at weekly intervals. If contaminants are found, the desired fungus can sometimes be saved with a serial transfer to a fresh medium. When making these transfers a thick cultivation loop or a sterile, sharp scalpel are useful.

III. Maintenance methods

The traditional maintenance methods for ectomycorrhizal fungi are agar slant cultures and the symbiotic synthesis of ectomycorrhizal fungi with the macrosymbiotic plant. There are also other more or less successful methods that will be described here.

A. Agar slant cultures

Amongst the agar media which can be used for maintenance of slant cultures are modified Hagem's and modified Melin–Norkrans' agar (described in Section II.A) with or without the addition of aureomycin. Good growth has also been obtained using the low pH mycological agar, which can be obtained commercially (Difco Laboratories, 1984). It contains soytone 10.0 g, glucose 10.0 g, agar 15.0 g and water 1000 ml and pH adjusted to 4.8 ± 0.2. We have adjusted the pH with HCl instead of lactic or acetic acid. Many fungi grow well on the medium of Marx–Melin–Norkrans (Marx, 1969) containing $CaCl_2$ 0.05 g, NaCl 0.025 g, KH_2PO_4 0.5 g, $(NH_4)_2HPO_4$ 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $FeCl_3$ (1%) 1.2 ml, thiamine HCl 100 μg, malt extract 3 g, sucrose 10 g, agar 15 g and 1000 ml distilled water.

Test tube cultures save space and can be held for 3–6 months in a cold room (3–4 °C). Molina and Palmer (1982) have used cultivation tubes containing 3 ml of agar medium per tube but we use 5–10 ml medium per tube in order to minimize the effects of drying. Standard test tubes (16 mm × 160 mm or 20 mm × 150 mm) with straight rims and aluminium Cap-O-Test or Labocap caps are useful. Molina and Palmer (1982) recommended serial transfers every third or fourth month but Marx and Daniel (1976) and M. Lindeberg (pers. commun.) transfer them at 5- or 6-monthly intervals. A minimum of four tubes should be inoculated and stored in at least two refrigerators at 3–4 °C.

Ectomycorrhizal cultures have been saved on agar slants for years or even decades, but some of these cultures have altered characters after long preservation in the laboratory. Most importantly, they may be unable to form mycorrhiza with plant symbionts, although there might still be significant stimulation of the growth of host plants (Sen, 1990). After many serial transfers some strains will inevitably be lost.

B. Sterile water

The use of sterile water for the maintenance of ectomycorrhizal fungi, originally described by Marx and Daniel (1976), has been found in our

laboratory to be a very valuable method that saves both labour and materials (Heinonen-Tanski, 1989). The maintenance times tested by us varied from 6 to 24 months. This procedure preserved the growth of all strains (over 50 tested, half from old collections and half recently isolated from Finnish forests, mainly *Cenococcum geophilum*, *Pisolithus tinctorius* and different *Suillus* spp.) with the exception of one old collection strain of *Paxillus involutus*, which was unable to grow further on agar slants, and two *Suillus* strains that gave modest growth (Table I). For this procedure the only requirements are young cultures, sterile water in screw cap bottles and a refrigerator.

The bottles used are autoclavable reagent bottles (50–150 ml) containing respectively 30 and 100 ml water sterilized for 15 min at 121 °C. Smaller bottles are more useful, because it is easier to take slippery growth pieces from them for further studies.

Pieces of inocula from the edges of young ectomycorrhizal colonies growing on Petri dishes containing modified Hagem's or modified Melin–Norkrans' agar are removed with a thick loop, scalpel or sterile cork borer. The size of a piece is approximately 5 mm × 5 mm. Between 4 and 6 pieces can be added to the same bottle, which is placed in a refrigerator (3–4 °C).

Recently Richter and Bruhn (1989) using similar methods reported that only about a half of their tested mycorrhizal strains survived after

TABLE I
Viability of ectomycorrhizal fungi after different maintenance methods

Method	Number of strains (and percentage in parenthesis)		
	Good growth	Modest growth	No growth
Sterile water at least 24 months	41 (93)	3 (7)	0 (0)
Sterile water at least 6 months[a]	3 (50)	2 (33)	1 (17)
Silica gel	15 (42)	1 (3)	19 (54)
Sand	29 (66)	4 (9)	11 (25)
Garden soil	2 (7)	0 (0)	24 (92)
Slants at −20°C without protectant	4 (16)	5 (20)	16 (64)
Slants at −20°C with 20% glycerol	4 (17)	1 (4)	18 (78)
Slants at −20°C with 20% mannitol	3 (13)	1 (4)	19 (83)

[a]No tests in sterile water during 24 months.

2–4 years in sterile water. It is important that research is done to discover the reasons for these contradictory results.

C. Maintenance at −20 °C, in silica gel, or in sand or soil

The maintenance methods described for fungi by Smith (1984) have also been tested on ectomycorrhizal fungi (Heinonen-Tanski, 1989). These methods were: (a) agar slants at −20 °C with or without addition of a protection agent (20% glycerol or mannitol); (b) maintenance in sterile, dried silica gel granules, where mycelium suspension in a minimal volume of milk was added to bottles containing sterile, frozen silica gel at 4 °C and shaken daily in closed bottles; (c) maintenance in thrice heat-sterilized sand or garden soil moistened with sterile water to 20%, where mycelium was added to the medium and allowed to grow for one week at room temperature before storage at 5 °C (see Table I). Growth was monitored after one month.

As can be seen from Table I, the maintenance methods involving deep-freezing, silica gel and garden soil were unsuccessful, giving either very weak or no growth. Only some *Cenococcum geophilum* strains survived. These methods therefore cannot be recommended. Sand preservation gave better results (29 strains of 44 survived), but was not efficient enough to be recommended for general use.

D. Maintenance as a grain culture

If storage for a relative short period (less than one year) is required, the grain culture technique may be helpful. Park (1971) and Göbl (1975) have found this method suitable for the preservation of some ectomycorrhizal fungi for up to 9 months when making inoculum preparations. Liquid medium inoculum is introduced into a bottle or into autoclavable plastic bags containing cooked and sterilized wheat or other cereal grains with the addition of some calcium carbonate or calcium sulphate. The fungi are allowed to grow and the containers are shaken gently once a week. The cultures developed can then be stored in a cold room for up to nine months. The value of this simple and cheap method as a maintenance method should be carefully determined for different types of ectomycorrhizal fungi.

Grain cultures as forest plantation inoculants have also been used by other research workers as reviewed by Marx (1980) although these were not always found to give good results.

E. Maintenance at extra-low temperatures

Cell material has in many cases been preserved at −75 to −80 °C or in liquid nitrogen. Experience of such methods for maintaining ectomycorrhizal fungal strains is as yet insufficient for any recommendations to be given.

F. Maintenance of ectomycorrhizal fungi in symbiosis

The maintenance of ectomycorrhizal fungi with their macrosymbiotic plant partners is the most natural way and is to be recommended, at least for the most valuable strains. These synthesized cultures can be either true diaxenic or only semi-aseptic. Synthesis is also useful in rejuvenating strains. The fungi can be reisolated from mycorrhiza or from sporophores (Marx, 1980). Synthesis methods are described in detail by Peterson and Chakravarty (Chapter 3, this volume).

IV. Conclusions

To reduce labour and to allow comparisons between old cultures and fresh isolates, safe, easy, economical and ecologically acceptable methods are needed for maintaining ectomycorrhizal fungi. The physiological diversity of these fungi means that they can adapt themselves to very different environments. Thus, for example, *Cenococcum geophilum* was resistant to most of the maintenance methods tested by us and was also found to be relatively resistant to industrial air pollutants (Holopainen and Vaittinen, 1988). Conversely *Suillus variegatus* was quite sensitive to most of the maintenance methods tested and also to air pollutant exposure (Ohtonen *et al.*, 1990).

A single method may not always be the most suitable for maintaining many different ectomycorrhizal strains. The method selected will depend on the facilities available in a particular laboratory. For maintenance of a small collection serial transfer would be the simplest solution, although the strains are susceptible to genetic changes; a risk which could be reduced by making regular plant–mycorrhiza syntheses. In larger collections if there is a shortage of laboratory technicians the suitability of sterile water or maybe oil as maintenance methods should be investigated.

There is a pressing need for further development of safe and reliable maintenance methods for ectomycorrhizal fungi. Reports of successful and unsuccessful methods are welcome to help us in developing the

utilization of ectomycorrhizal fungi in tree cultivation and for protecting forests against environmental stresses.

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